Master's Thesis

Effects of the novel compound DK223 on migration of human keratinocytes

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GRADUATE SCHOOL

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ABSTRACT

Wound healing plays an important role in protecting the human body from inflammation or infection. Cell migration and proliferation of keratinocytes is essential for proper wound healing. Recently, several studies have demonstrated that secondary compounds produced in certain plants could affect keratinocyte migration *in vitro* and *in vivo*. In this study, we identified a novel compound DK223 ([1*E*,2*E*-1,2-bis(6-methoxy-2*H*-chromen-3-yl)methylene]hydrazine) that induced human keratinocyte migration. We evaluated the regulation of migration-associated proteins, such as E-cadherin and vimentin, in human keratinocytes. DK223 up-regulated keratinocyte migration and significantly increased the epithelial marker E-cadherin in a time-dependent manner. We also found that reactive oxygen species (ROS) increased significantly in keratinocytes after 2 hours of DK223 exposure, returning to normal levels after 24 hours, whick indicated that DK223 had an early shock effect on ROS production. In conclusion, DK223 induced keratinocyte migration via ROS production, indicating a potential use for DK223 in the wound healing process.

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

HaCaT, immortalized keratinocyte cell line

RPMI, Rosewell Park Memorial Institute

FBS, fetal bovine serum

PAA, penicillin/streptomycin

DMSO, dimethyl sulfoxide

ECIS, electric cell-substrate impedance sensing

MTT, 3[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide

RIPA, radioimmunoprecipitation assay

BCA, bicinchoninic

SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis

RT-PCR, reverse transcription polymerase chain reaction

PBS, phosphate buffered saline

ROS, reactive oxygen species

DCFH-DA, 2'7'-dichlorofluorescin diacetate

MET, mesenchymal-epithelial transition

EMT, epithelial-mesenchymal transition

DPI, diphenylene iodonium

NAC, N-acetylcysteine

MMPs, metalloproteinases

ECM, extracellular matrix

I. INTRODUCTION

As the outer sheath of the human body, the skin absorbs initial damage from injuries or wounds. Disruption of the normal anatomic structure and loss of organ function lead to the repair process known as wound healing. Wound healing integrates different types of dermal cells, such as keratinocytes and dermal fibroblasts, to mediate hemostasis, coagulation, inflammation, proliferation and remodeling [1,2]. Wound healing begins with inflammation and immune cell recruitment to protect tissue from foreign invaders, such as bacteria and viruses. The next phase resurfaces the skin by stimulating regeneration and repair through proliferation of fibroblasts, re-epithelialization of keratinocytes, and other mechanisms. The final phase involves scar formation, which can cause concern for some patients when the scar is not aesthetically pleasing. Therefore, several studies have investigated methods to enhance the regeneration process, which is a specific substitution of the damaged tissue, and to reduce skin repair, which causes fibrosis and scar formation [1].

Herbal plants, such as garlic and curcuma, have been used traditionally for wound healing, and it is known that their effects are due to secondary compounds, such as flavonoids, saponins and alkaloids. However, these coumpounds possess anti-inflammatory, anti-viral, and anti-oxidant capabilities, usually associated with impaired wound healing. For example, Lopez-Jornet et al. showed that potassium apigenin and other flavonoids present in verbena extract possess powerful anti-inflammatory properties [3]. In addition, Duarte et al. demonstrated the use of chamomile extract ointment for stimulating oral mucosa re-epithelialization and formation of collagen fibers after 10 days of treatment [4].

Novel compounds have been synthesized using innovative chemical techniques that expand the origin conformation. In this study, we examined the hypothesis that the novel compound DK223 ([1*E*,2*E*-1,2-bis(6-methoxy-2*H*-chromen-3-yl)methylene]hydrazine) could induce keratinocyte migration, anti-aging gene expression, and enhance the regeneration of skin, rather than mediate scar formation, *in vitro* and *in vivo*.

II. MATERIALS AND METHODS

Reagent

The DK223 (Figure 1) compound was synthesized and kindly provided by Youngho Lim in the Division of Bioscience and Biotechnology, Konkuk University, Seoul, Korea.

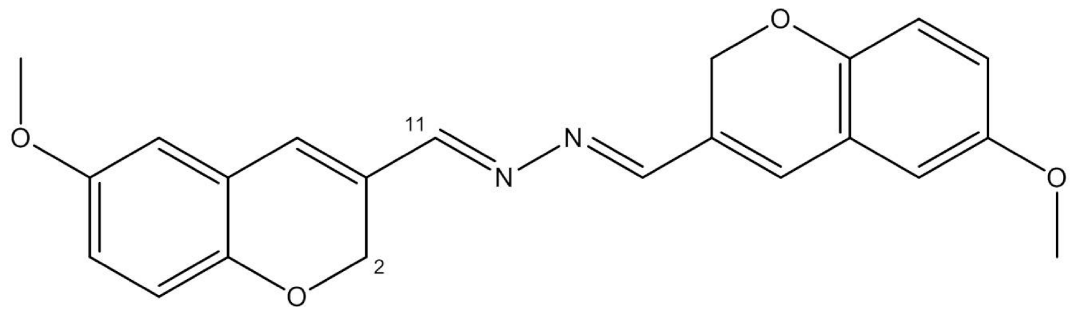
Cell culture

In this study, we used the spontaneously immortalized keratinocyte cell line (HaCaT). HaCaT cells were cultured in RPMI media (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin/streptomycin (PAA). Cells were incubated in a humidified atmosphere at 37℃ with 5% CO_2.

Scratch wound healing assay

HaCaT cells were seeded in a 48-well plate before a 24-h wound healing scratch assay [5]. The scratch wound was made using a sterile 200-㎕ pipette tip to scratch a line across the bottom of the culture dish. Culture media was then removed and replaced with fresh media supplemented with DK223 or dimethyl sulfoxide (DMSO) (Amresco) as a control. Photographs were taken at 4x magnification using and OLYMPUS IX70 microscope equipped with a digital camera before treatment and 48 h after treatment. The length of the scratch was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA), and the difference

between the initial and final widths was calculated.



(1*E*,2*E*)-1,2-bis((6-methoxy-2*H*-chromen-3-yl)methylene)hydrazine

Figure 1. Structure of DK223

Migration assay with the electric cell-substrate impedence sensing (ECIS) system

Electrode plates were induced with an electrode-stabilizing solution provided by the manufacturer 10 min prior to seeding with HaCaT cells overnight. Cells were then treated with different concentrations of DK223. After 24 h, the electrode plates were placed in the ECIS apparatus in the incubator for migration analysis using the ECIS system software, according to the manufacturer's instructions (Applied Biophysics).

MTT assay

Cells were seeded in a 96-well plate at a density of 3 x 10^4 /ml. HaCaT cells were treated with DK223 or DMSO for 24 h, while dermal fibroblasts were treated for 48 h. MTT solution (Amresco, 5 mg/ml) was added to each well and incubated at 37℃ for 4 h. The medium was then gently removed and replaced with 150 ㎕ DMSO, followed by shaking for 30 min to dissolve the precipitate, and the absorbance was measured at 570 nm.

Western blot

Cells were treated with DK223 or DMSO in a time- and dose-dependent manner. Cells were then harvested using a cell scraper and lysed in RIPA buffer. The protein concentration was determined using a bicinchoninic (BCA) assay (Thermo Scientific). The proteins were separated by SDS-PAGE, followed by Western blot

using the appropriate antibodies.

All antibodies for Western blot were purchased commercially and prepared according to the manufacturer's protocol. p21 (2947), AKT (9272) phosphorylated AKT (9271S), ERK (4695), Slug (9585S), and GAPDH (2118) were obtained from Cell Signaling. E-cadherin (610181) was purchased from BD Science Transduction. Cyclin E (sc-247), cyclin D1 (sc-246), p53 (sc-126), phosphorylated ERK (sc-7383), collagen I (sc-25974), collagen III (sc-28888), and fibronectin (sc-9068) were purchased from Santa Cruz Biotechnologies. Differences were confirmed using Image J software to determine the relative ratio of changes in the target protein to those of the DMSO control.

RT-PCR

Total RNA from treated cells was extracted using Trizol reagent (Invitrogen). Equal amounts of total RNA in each treatment group were used to synthesize cDNA using a reverse transcriptase kit (Promega). The resulting cDNA was used for RT-PCR using the G-taq kit (Cosmo Genetech) based on the manufacturer's protocols.

RT-PCR was performed using the following gene-specific primers designed using the Blast Prime website: GAPDH, forward primer 5'-GAAGGTGAAGGTCGGAGTC-3', reverse primer 5'-GAAGATGGTGATGGATTTC-3'; p53, forward primer 5'-ACACGCTTCCCTGGATTGG-3', reverse primer 5'-CTGGCATTCTGGGAGCTTCA-3'; p21, forward primer 5'-GTCAGTTCCTTGAGCCG-3', reverse primer 5'-GAAGGTAGAGCTTGGGCAGG-3'. Image J software was used to analyze the results and the relative ratio of changes in the target gene to those of the DMSO

control.

Flow cytometry for cell cycle analysis

For cell cycle analysis, exponentially growing cell cultures were incubated for 24 h in the presence or absence of DK223. Cells were then trypsinized and centrifuged, and the cell pellets were immediately fixed with ice-cold 70% ethanol. Cells were washed twice with PBS containing 2 mM EDTA to remove ethanol and incubated with propidium iodide for 30 min at 37℃. Cells were analyzed by flow cytometry using a FACSCalibur 1 (Becton Dickinson) [6].

ROS generation analysis

Production of ROS was measured based on ROS-dependent oxidation of the oxidation-sensitive fluorescent probe 2'7'-dichlorofluorescin diacetate (DCFH-DA) to DCF [7]. Cells were seeded in a 96-well plate (3 x 10^4 cells/ml) for 24 h before flavonoid treatment and then treated with 2 μM DK223 for 0, 0.5, 1, 2, 12, and 24 h. H_2O_2 was used as a positive control. The cells were washed with PBS before removing medium, 200 ㎕ of DCFH-DA (100 μM in PBS containing 1% FBS) were added, and the cells were incubated for 30min at 37℃ in the dark. Intracellular ROS production was measured using DCFH-DA with a DCF-DA microplate assay. A spectrofluorometer (SPECTRAFLUOR, Tecan) was used to assess ROS generation by measuring the fluorescence intensities from 10,000 cells/well at an excitation wavelength of 485 nm and a emission wavelength of 530 nm.

III. RESULTS

When an injury occurs, a series of complex intracellular mechanisms and pathways is activated and coordinated. Normally, wound repair processes can be characterized into three phases: inflammation, new tissue formation, and remodeling. Tissue formation occurs a few days after injury and is characterized by proliferation and migration of different cell types, initiated by the migration of keratinocytes as the primary dermal cell type [2]. Thus, we initially treated the human keratinocyte HaCaT cell line with DK223 and investigated the mRNA and protein expression of migration-related genes.

DK223 promoted the migration but not the proliferation of HaCaT cells

To investigate the ability of DK223 to induce migration of human keratinocytes, we treated HaCaT cells with different concentrations of DK223 in a 48-h scratch wound-healing assay. The results showed that DK223 at 2 μM significantly enhanced migration of HaCaT, whereas other concentrations did not produce marked effects (Figure 2A). In order to confirm these results, we performed the migration assay with the ECIS system using other concentrations of DK223. In this assay, during cell growth the current is impeded based on the number of cells covering the electrode, which indicates the velocity of cell movement. The results were collected as impedance versus time. As expected, 2 μM DK223 significantly enhanced the migration velocity of keratinocytes after 24 h compared with the control (Figure 2B).

We used the MTT assay to assess proliferation of HaCaT cells in response to DK223, and the results demonstrated that there was no significant change in

keratinocyte growth with 2 μM DK223 (Figure 3A). As expected, the mRNA and protein expression of p53, p21, p27, cyclin D, and cyclin E did not significantly change compared with the untreated cells (Figure 3B). These observations indicate that DK223 could induce cell migration but not proliferation of human keratinocytes.

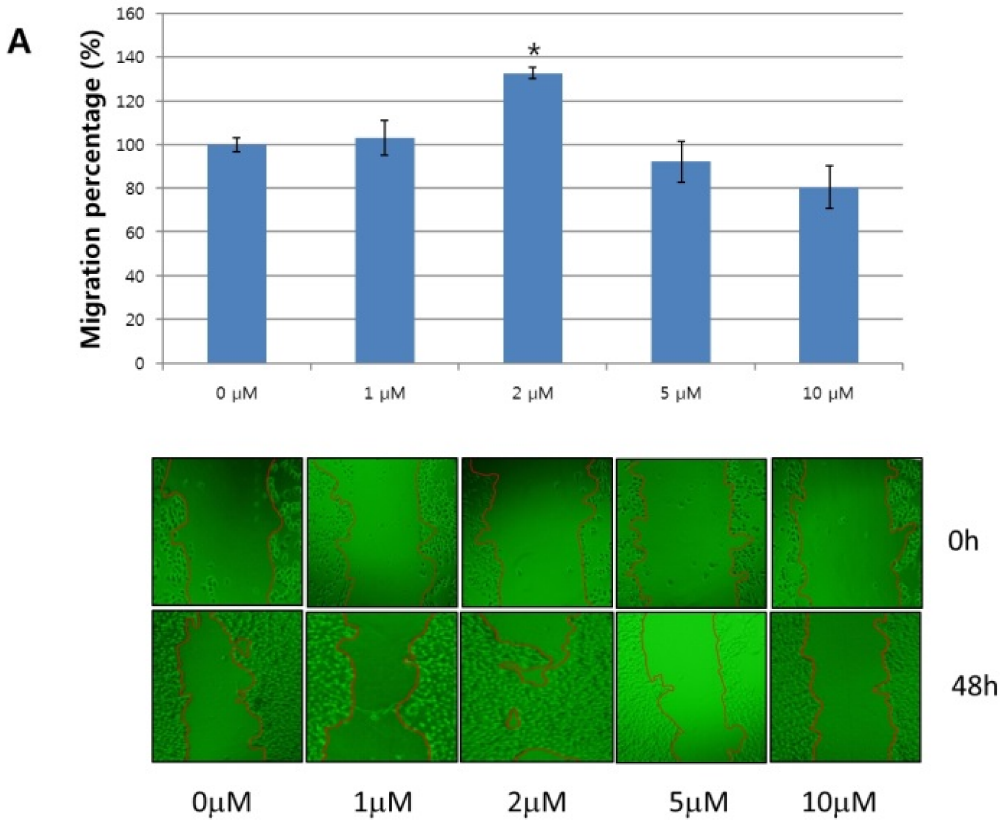


Figure 2A. Effect of DK223 on migration of HaCaT cells. Cells were seeded at a density of 3 x 10^4/ml 24 h before the scratch test. Scratch distances were measured 48 h after DK223 treatment. Data represent the mean distance percentage ±SD for at least three replicates, *P < 0.05.

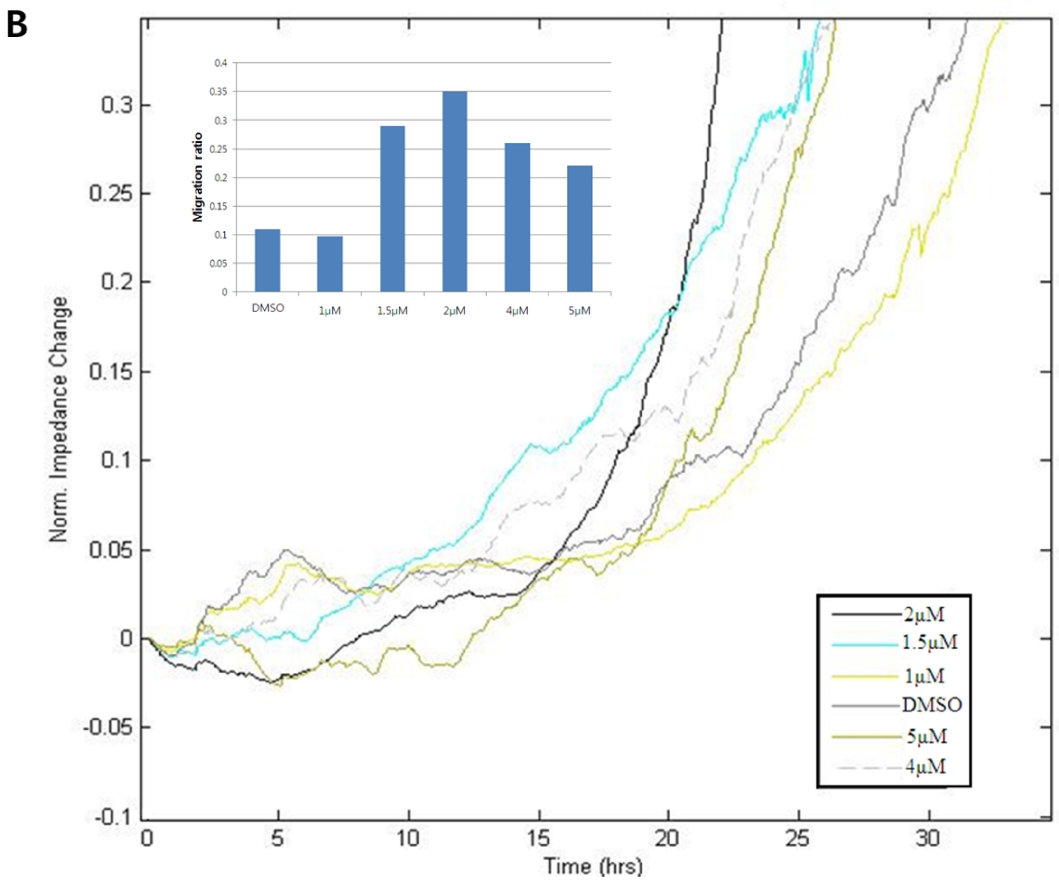


Figure 2B. Migration velocity of HaCaT cells. Cells were seeded at a density of 3 x 10^4/ml 24 h before treatment. The impedances were measured and analyzed at 24 h by the ECIS system software. The line data indicate the migration velocity of cells treated with different concentrations of DK223.

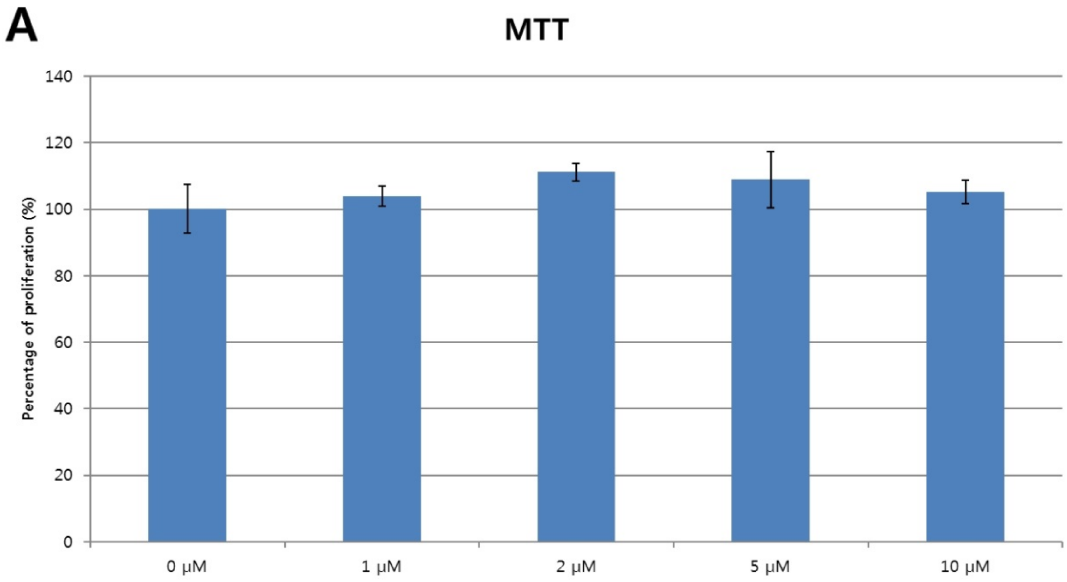


Figure 3A. DK223 treatment did not affect HaCaT cell proliferation. Cells were seeded overnight before treatment with varying concentrations of DK223 for 24 h. Cells were then incubated with MTT for 4 h at 37℃, and the absorbance was measured at 570 nm.

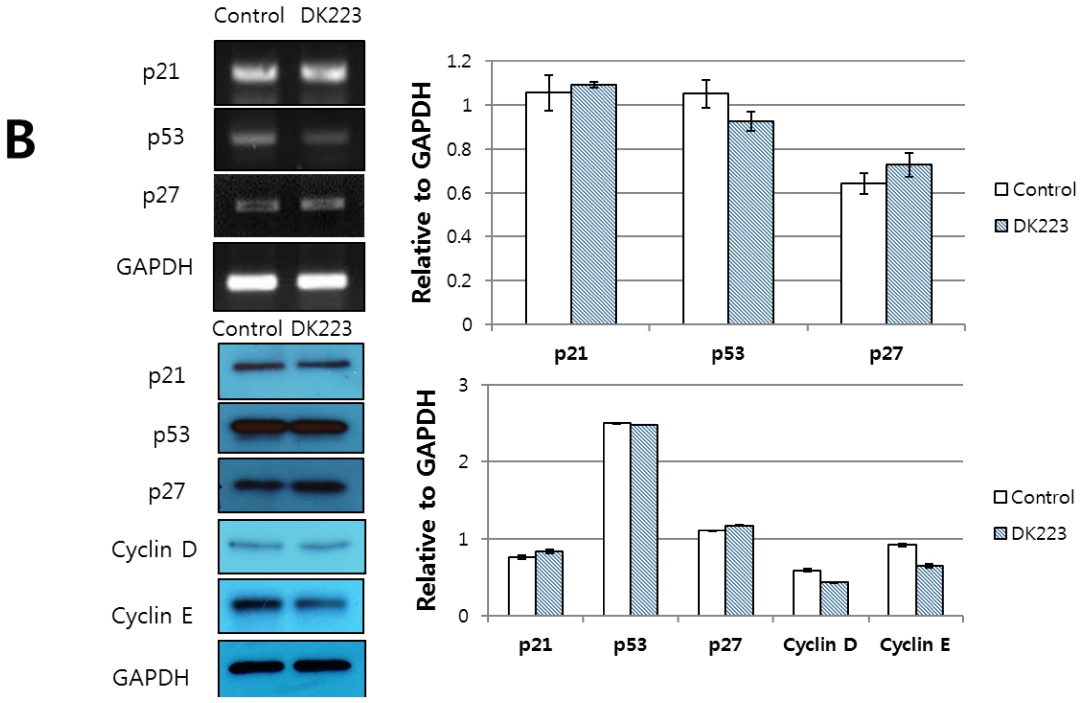


Figure 3B. DK223 treatment did not affect HaCaT cell cycle protein expression. The expression of cell cycle-related proteins in HaCaT cells induced by 2 μM DK223 for 24 h. Protein expression was examined by Western blot analysis with the specific antibodies indicated, and mRNA expression was assessed by RT-PCR with the gene-specific primers indicated. A histogram depicting the ImageJ data analysis results is shown. Values represent means ±SD of three independent experiments. *P < 0.05 compared with control.

DK223 induced mesenchymal-epithelial transition (MET) but not epithelial-mesenchymal transition (EMT) in HaCaT cells

EMT is critical for wound healing, and tissue regeneration has been shown to induce keratinocyte migration [8]. We examined the mRNA and protein expression of EMT markers, such as vimentin, E-cadherin, and Slug after DK223 treatment to investigate whether DK223-induced migration affected the EMT process. As shown by Western blot in Figure 4A, two mesenchymal markers, vimentin and Slug, were down-regulated in a time-dependent manner, whereas the adherens junction protein E-cadherin was increased significantly, peaking 24 h after treatment. These results correlated with the changes in mRNA expression measured by RT-PCR (Figure 4B). This suggested that DK223 induced MET, rather than EMT, processes in HaCaT cells.

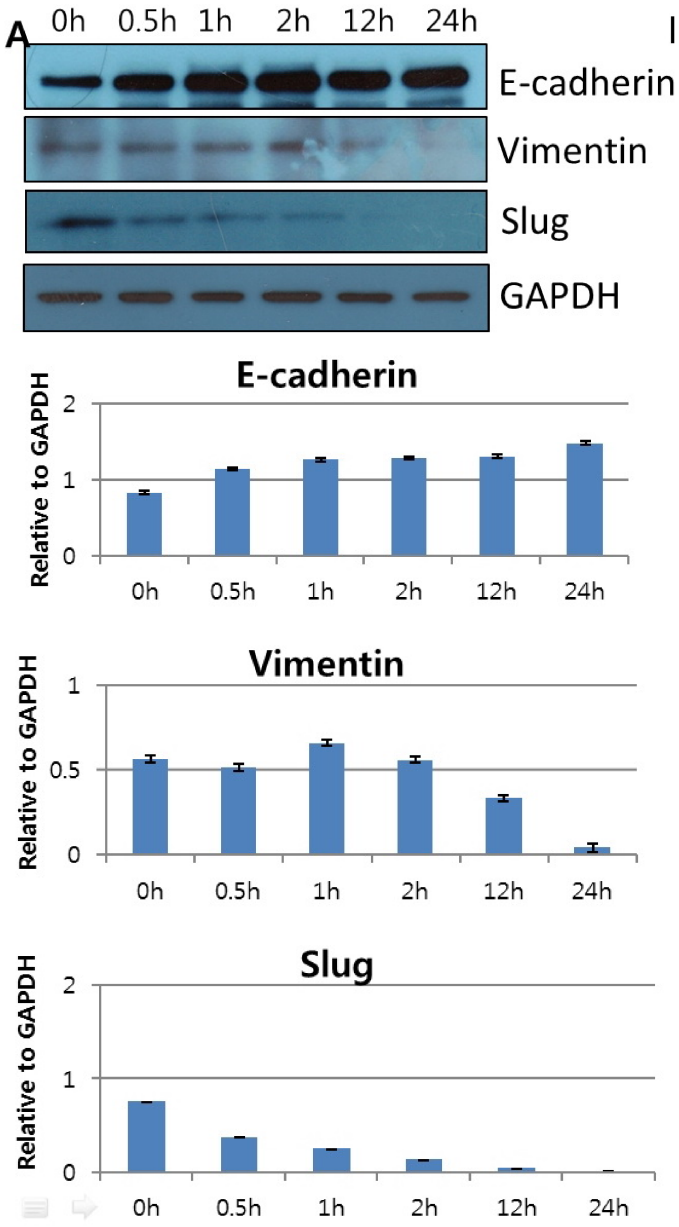


Figure 4A. Time-dependent protein expression of EMT-related genes after treatment with DK223. Protein expression was examined in HaCaT cells treated with 2 μM DK223 for different time periods using Western blot analysis with the specific antibodies indicated.

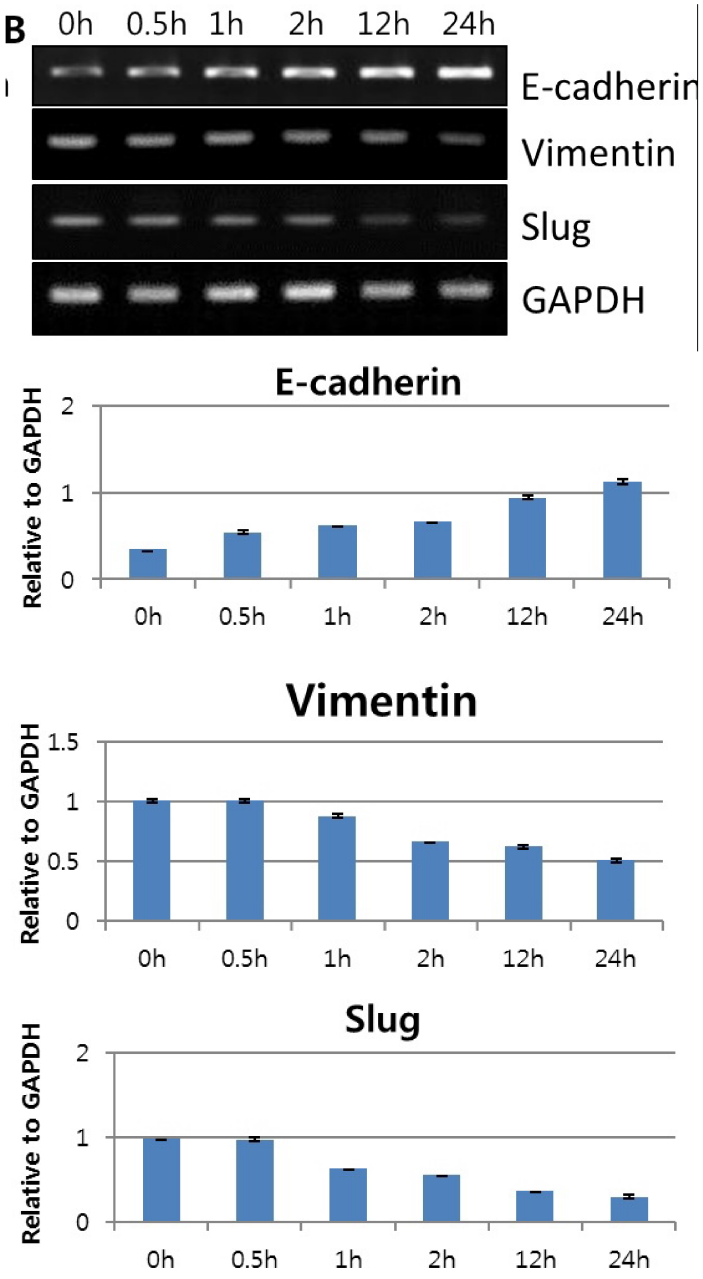


Figure 4B. Time-dependent mRNA expression of EMT-related genes after treatment with DK223. mRNA expression was investigated by RT-PCR with the specific primers indicated. A histogram shows the results of ImageJ data analysis. Values represent means ±SD of three independent experiments.

DK223 induced NADPH oxidase expression and ROS production

To gain insight into the mechanism of keratinocyte migration induced by DK223, we assessed NADPH oxidase expression and ROS production, as well as the signaling pathways related to cell migration. As demonstrated in Figure 5A, NADPH oxidase 4 mRNA and protein expression increased in a time-dependent manner, peaking 2 hr after treatment. This correlated with an up-regulation in ROS production, which also peaked at 2 h (Figure 5B), as well as with the effects on cellular signaling pathways. Phosphorylation of Akt was down-regulated at an early time point, while phosphorylation of ERK and JNK increased after 30 min of treatment (Figure 5C). These findings suggest a relationship between the early increase in ROS production at 2 h and concomitant changes in intracellular signaling pathways.

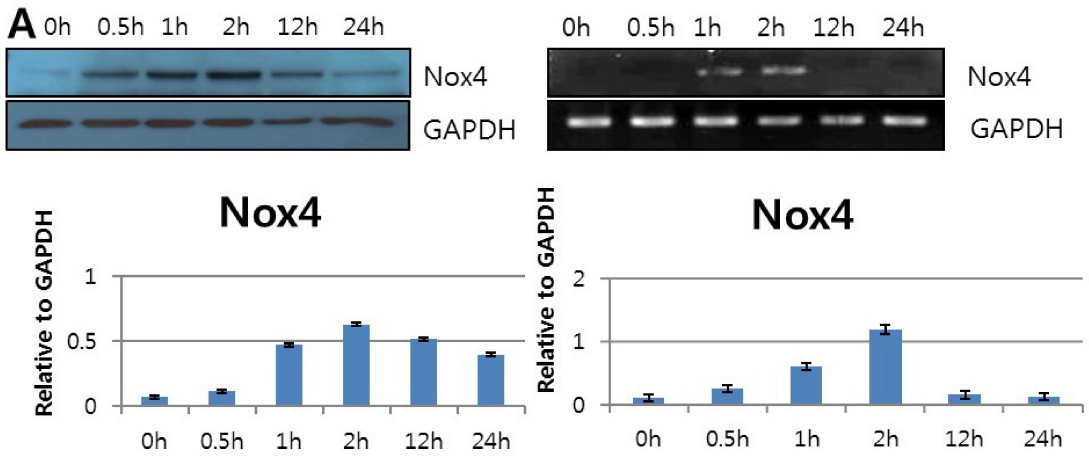


Figure 5A. NADPH oxidase in HaCaT cells treated with DK223. The expression of NADPH oxidase 4 was examined by Western blot and RT-PCR as described previously. A histogram shows the results of ImageJ data analysis.

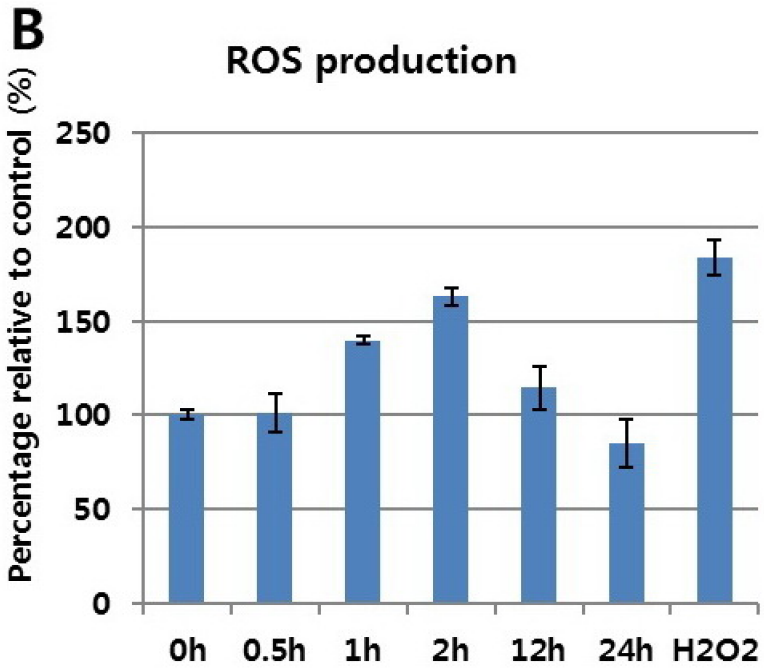


Figure 5B. ROS production in HaCaT cells treated with DK223. Time-dependent ROS generation was detected in treated HaCaT cells using the DCF-DA assay. Cells were treated with 2 μM DK223 at different times and then stained with DCF-DA to detect ROS generation, as described previously. H_2O_2 was used as a positive control. A histogram shows the results of ImageJ data analysis.

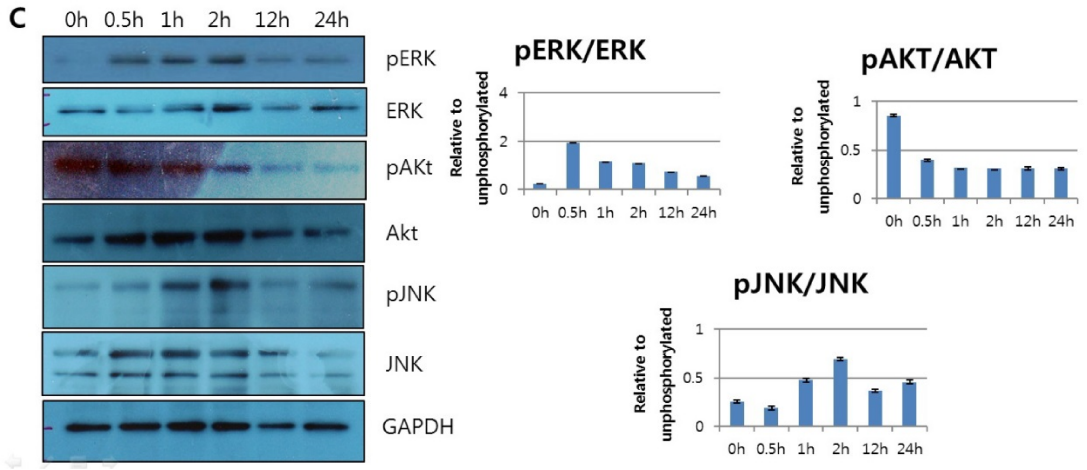


Figure 5C. Signaling pathways in HaCaT cells treated with DK223. Signaling pathways were examined by Western blot with the specific antibodies indicated. A histogram shows the results of ImageJ data analysis.

DK223 affected signaling pathways via ROS production

To explore the mechanism mediating the effects of DK223 on signaling pathways and ROS production, we pretreated cells with the NADPH inhibitor diphenylene iodonium (DPI) or the ROS scavenger N-acetylcysteine (NAC) prior to treatment with DK223 for 2 h. As expected, NADPH oxidase 4 was inhibited by DPI and NAC pretreatment. NAC also reduced DK223 induced phosphorylation of JNK and ERK. Interestingly, the activation of Akt, which was down-regulated by DK223 at 2 h, was also attenuated significantly by NAC and DPI (Figure 6). These results indicated that DK223 induced the phosphorylation of JNK and ERK at 2 h via ROS production.

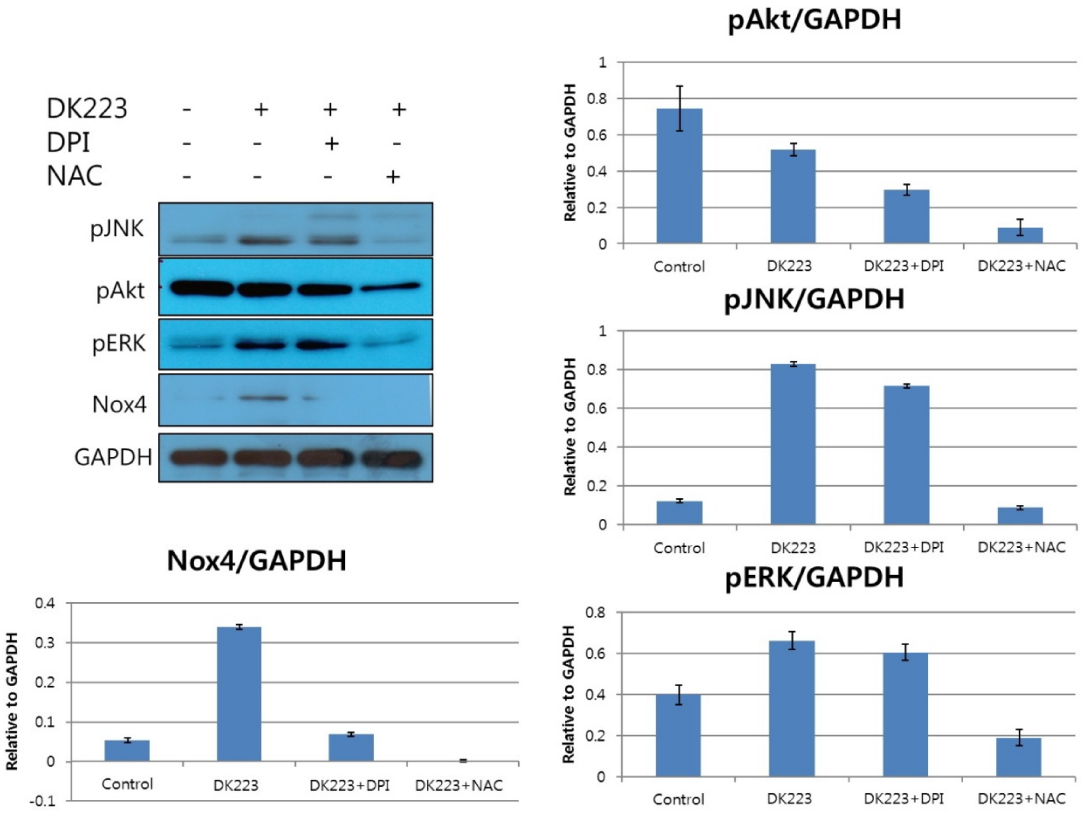


Figure 6. DPI and NAC pretreatment inhibited NADPH oxidase 4 and activation of ERK and JNK pathways. Cells were pretreated with 10 μM DPI or 20 μM NAC for 2 h before 24-h DK223 incubation. Signaling pathways were examined by Western blot with the specific antibodies indicated. A histogram shows the results of ImageJ data analysis.

Migration-related proteins are down-regulated by DK223 treatment in HaCaT cells

Because the EMT process did not appear to involve DK223-induced migration, we investigated other migration-related proteins, including the metalloproteinases (MMPs), collagen I and III. It have been demonstrated recently that these proteins could regulate the migration and proliferation of keratinocytes [9,10]. We observed a significant down-regulation in the mRNA and protein expression of collagen I and III after 24 h of DK223 treatment (Figure 7A,B). Thus, high levels of proteinases might degrade collagen I and III upon DK223 treatment. Moreover, another ECM protein, fibronectin, was highly expressed (Figure 7A,B) with DK223 treatment.

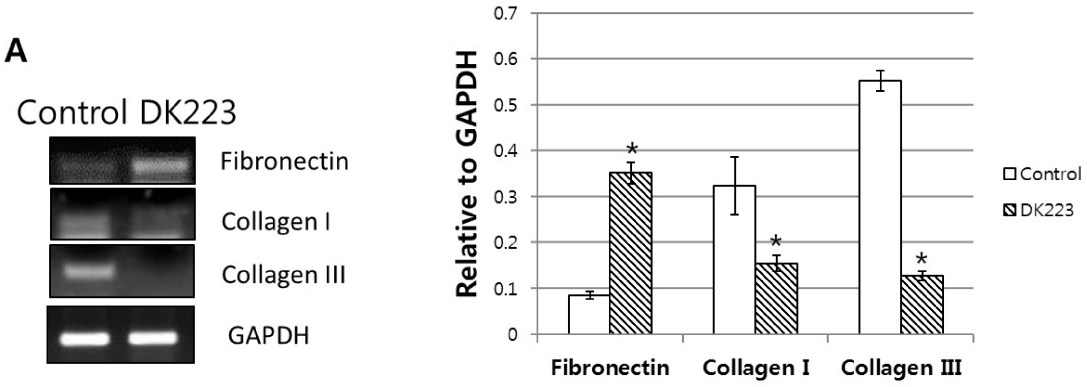


Figure 7A. mRNA expression of extracellular matrix proteins in HaCaT cells treated with DK223. The expression was examined in HaCaT cells treated with 2 μM DK223 for 24 h using RT-PCR with the specific primers indicated. A histogram shows the results of ImageJ data analysis. Values represent the means ±SD of three independent experiments. *P < 0.05 compared with the control.

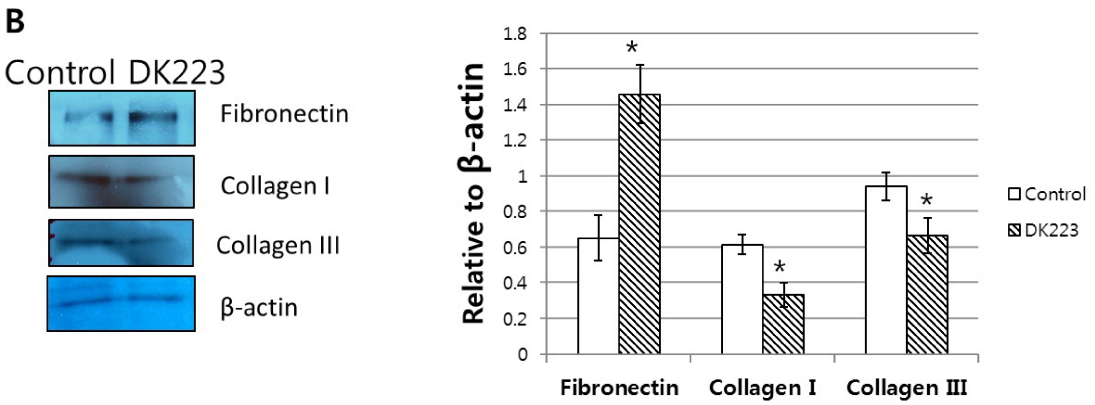


Figure 7B. Protein expression of extracellular matrix proteins in HaCaT cells treated with DK223. Cells were treated with 2 μM DK223 in serum-free media. Conditioned media were removed and concentrated using Amicon centrifugation. Proteins in the media were analyzed by Western blot for collagen I, III, and fibronectin using the specific antibodies indicated. A histogram shows the results of ImageJ data analysis. Values represent the means ±SD of three independent experiments. *P < 0.05 compared with the control.

IV. DISCUSSION

The wound repair process protects the human body from infections and fluid loss; yet, it results in scar formation. In this study, we investigated the effects of DK223 on wound healing processes *in vitro* and *ex vivo* with the expectation that DK223 could induce skin regeneration.

Skin regeneration is characterized by a re-epithelialization process that covers the wound surface and stimulates re-angiogenesis [11]. After the wound has been blocked by a blood clot, fibroblasts begin secreting ECM proteins, such as collagen III and fibronectin. These proteins reorganize the base structure under the blood clot to aid in keratinocyte migration [1]. We have studied this phenomenon by treating human keratinocytes with different concentrations of DK223 *in vitro*. While DK223 did not promote keratinocyte cell growth, migration was enhanced significantly. Mesenchymal markers, such as vimentin and Slug, were down-regulated in a time-dependent manner, whereas the epithelial marker E-cadherin was increased markedly. This indicates that keratinocytes induced MET and that the cells were more tightly bound together as migration increased. This was also reported by Li et al., who showed that a whole sheet of epithelial cells could migrate while maintaining tight cell-cell adhesion, a phenomenon they called “collective migration”. As shown in Figure 4, DK223 significantly up-regulated E-cadherin, which plays an important role in re-epithelialization, since adherens junctions prevent the epithelial barrier from further damage after wounding [12]. This indicates that DK223 was able to induce collective migration to protect the epithelial barrier from wounds.

In a recent study, Kim et al. demonstrated that HaCaT keratinocyte migration was induced by the EGF-like ligand neuregulin, which was activated via Rac1 and Nox-

driven ROS production [13]. In our study, DK223 also significantly increased HaCaT cell migration via intracellular signaling pathways. Our results demonstrated that ROS production peaked at 2 h, along with signaling proteins, such as Akt and ERK. In recent studies, Akt2 was shown to induce cell migration [14] and ERK/MAPK phosphorylation might be activated by ROS [15,16]. In addition, ERK/MAPK phosphorylation ameliorated keratinocyte migration [17]. In light of these results, we propose that DK223 induces keratinocyte migration by modulating intracellular signaling pathways via ROS production.

Once the wound has formed a blood clot via fibrin, dermal fibroblasts increase in proliferation and infiltrate under the wound. These cells secrete collagen and fibronectin, which replace the blood clot fibrin to accelerate wound closure [18]. Nevertheless, ECM accumulation in instances of excessive healing may cause unaesthetic scars [2]. In this study, DK223 induced keratinocyte migration to help the wound heal more rapidly, whereas it modulated ECM production in kerationcytes.

V. CONCLUSION

This study demonstrated that the novel compound DK223 induced keratinocyte migration, while maintaining balance among ECM proteins, such as collagen I, III, and fibronectin. It has potent capabilities in wound healing and skin regeneration.

VI. REFERENCES

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